INSTITUUT VOOR PLANTENZIEKTENKUNDIG ONDERZOEK
WAGENINGEN, NEDERLAND
DIRECTEUR: Dr. J. G. TEN HOUTEN

MEDEDELING No 185 EN 186

# ONDERZOEKINGEN OVER VIRUSZIEKTEN VAN VLINDERBLOEMIGEN

## (STUDIES ON VIRUS DISEASES OF LEGUMES)

DOOR

J. P. H. VAN DER WANT EN L. BOS

# THE RED CLOVER VEIN-MOSAIC VIRUS IN THE NETHERLANDS

DOOR

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NSP
RAM
MM
abst 22.4.59

OVERDRUK UIT:
TIJDSCHRIFT OVER PLANTEZIEKTEN, 64:419–421, 1958 EN 65:13–23, 1959

COMMONWEALTH MYCOLOGICAL INSTITUTE
16 APR 1959

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T. Pl.ziekten 64 (1958): 419-421

# ONDERZOEKINGEN OVER VIRUSZIEKTEN VAN VLINDERBLOEMIGEN[1]

*With a summary: Studies on virus diseases of legumes*

DOOR

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## INLEIDING

Vlinderbloemige gewassen zijn in Nederland economisch en landbouwkundig van veel betekenis. Onder de ziekten, die deze gewassen belagen, nemen die, welke door virussen worden teweeggebracht, een belangrijke plaats in. Over de mate, waarin ze hier te lande optreden en de wijze, waarop ze kunnen worden bestreden of voorkomen, is nog betrekkelijk weinig bekend. Daarom worden sinds enige tijd op het Instituut voor Plantenziektenkundig Onderzoek onderzoekingen over de virusziekten van vlinderbloemigen verricht.

In de fytopathologische literatuur zijn verspreide gegevens te vinden over het voorkomen van virusziekten bij leguminosen. Schrijvers hebben zich tot taak gesteld een systematisch onderzoek te verrichten over de betrokken virussen en hun gegevens te vergelijken met die, welke in de literatuur voorkomen.

De literatuurlijst, die aan deze publikatie is toegevoegd, geeft een overzicht van de artikelen, die in de loop van de jaren over in Nederland bij vlinderbloemigen verricht virusonderzoek zijn verschenen.

## METHODEN VAN ONDERZOEK

Het onderzoek is er op gericht te komen tot een inventarisatie en identificatie van de in Nederland bij vlinderbloemigen voorkomende virussen. Daarnaast zal worden nagegaan welke de economische betekenis van elk der gevonden virussen in ons land is.

Op verschillende wijzen worden gegevens bijeengebracht. In de eerste plaats wordt materiaal verzameld door waarnemingen in praktijkpercelen van de onderhavige gewassen te verrichten. Ook inzendingen van de voorlichtingsdiensten zijn hierbij welkom. Voorts worden op verschillende plaatsen in het land proefveldjes aangelegd, waarop in kleine vakjes een vrij groot aantal soorten en rassen van Papilionaceeën worden geteeld. Deze proefveldjes dienen om eventuele, in de desbetreffende streek voorkomende virussen als het ware te vangen.

Zijn eenmaal isolaties gemaakt, dan worden de betrokken virussen in de kas op een vrij uitgebreid toetssortiment geïnoculeerd. In vele gevallen kunnen de inoculaties met behulp van sap van zieke planten worden verricht. Lukt dit niet, dan wordt getracht dit te doen door middel van verschillende soorten insekten, doorgaans bladluizen. Het toetssortiment is in zijn eenvoudigste vorm samengesteld uit: boon, tuinboon, erwt (enkele rassen), rode, witte en inkarnaatklaver. Een uitgebreider toetssortiment omvat bovendien, naast enige andere vlinderbloemigen, een aantal plantesoorten, behorende tot andere families, zoals tabak,

[1] Aangenomen voor publikatie 15 augustus 1958.

komkommer, *Gomphrena globosa* en *Chenopodium amaranticolor*. Het doel van deze kasproeven is tot een differentiatie van de geïsoleerde virussen te komen, aan de hand van verschillen in toetssortiment en verschillen in symptomen, terwijl tevens op deze wijze wordt getracht eventuele mengsels van virussen in de enkelvoudige componenten uiteen te leggen. Onderzocht wordt in hoeverre centrifugeertechnieken mede hierbij van dienst kunnen zijn.

Is op grond van dit werk met proefplanten de indruk verkregen, dat een isolatie uit niet meer dan één virus bestaat, dan worden de eigenschappen in vitro bepaald, te weten de verdunningsgrens, de inactiveringstemperatuur en de houdbaarheid in bij kamertemperatuur bewaard perssap. Tevens worden de morfologische eigenschappen (vorm en afmetingen) met de elektronenmicroscoop bestudeerd, alsmede de serologische hoedanigheden van het betrokken virus. Ook de eventuele overdracht door middel van insekten wordt nagegaan. Op deze wijze worden de eigenschappen van de afzonderlijke virussen zo goed mogelijk omschreven ten einde in het vervolg een snelle herkenning van het virus, dus een snelle diagnose van de ziekte, mogelijk te maken.

Onderzoek over de eventuele rol van insekten als overbrengers van de virussen dient tevens om een inzicht te krijgen in de wijze, waarop verspreiding te velde geschiedt.

Hieronder volgt een opsomming van de in Nederland inmiddels uit vlinderbloemigen geïsoleerde virussen en de hierdoor veroorzaakte ziekten.

| | |
|---|---|
| 1. heksenbezemvirus | – heksenbezemziekte (vergroening) klavers |
| 2. komkommermozaïekvirus | – verdorringsziekte gele lupine<br>verwelkings- en necroseverschijnselen erwt |
| 3. luzernemozaïekvirus | – luzernemozaïek |
| 4. nerfmozaïekvirus van rode klaver | – nerfmozaïek rode en andere klavers,<br>gedrongen-topziekte erwt |
| 5. *Phaseolus virus* 1 | – rolmozaïek boon |
| 6. *Phaseolus virus* 2 | – scherpmozaïek boon,<br>waarschijnlijk ook lupinemozaïek |
| 7. *Pisum virus* 1 | – „enation" mozaïek erwt |
| 8. *Pisum virus* 2 | – gewoon erwtemozaïek |
| 9. tabaksnecrosevirus | – stippelstreep boon |
| 10. topvergelingsvirus | – topvergeling erwt en tuinboon |
| 11. vroege verbruiningsvirus | – vroege verbruining erwt |
| 12. witte-klavervirus | – een nog niet voldoende gedefinieerde ziekte |

Een uitvoerige behandeling van deze virussen en virusziekten wordt door schrijvers gegeven in een artikel in het maandblad Landbouwvoorlichting.

## NABESCHOUWING

De schrijvers hopen binnenkort te beginnen met de publikatie van de gegevens van hun onderzoekingen aangaande een aantal uit vlinderbloemigen geïsoleerde virussen. Dit artikel dient hiertoe als een inleiding.

Zoals reeds werd opgemerkt wordt nagestreefd om de resultaten van dit onderzoek te vergelijken met gegevens, die in de loop van de jaren in buitenlandse publikaties zijn neergelegd. Evenals bij zovele andere onderzoekingen over plantevirussen doet zich ook hier de noodzaak gevoelen van internationale samenwerking ten behoeve van de identificatie van in verschillende delen der wereld voorkomende virussen. De schrijvers stellen zich voor dit te bereiken door het uitwisselen van gegevens aangaande waardplanten, eigenschappen in vitro en

morfologie. Van grote betekenis is tevens het uitwisselen van antisera tegen de betrokken virussen.

Als voorbeeld van deze internationale samenwerking zal binnenkort in het kader van deze reeks onderzoekingen een artikel worden gepubliceerd over het nerfmozaïekvirus van rode klaver in Nederland, dat in samenwerking met Prof. Dr. D. J. HAGEDORN uit Madison, Wisconsin, U.S.A., tot stand is gekomen.


## SAMENVATTING

Vanwege de betekenis van de virusziekten voor de cultuur van vlinderbloemige gewassen is op het Instituut voor Plantenziektenkundig Onderzoek een uitgebreid, systematisch onderzoek aan de gang met als doel de inventarisatie en identificatie van bij deze gewassen in Nederland voorkomende virussen. De reeds geisoleerde virussen worden opgesomd. De literatuurlijst geeft een overzicht van datgene wat tot dusver in Nederland over dit onderwerp is gepubliceerd.

Het artikel vormt een inleiding tot een reeks publikaties, die betrekking zullen hebben op de verschillende virussen en de ziekten, die er door worden teweeggebracht.



## SUMMARY

Since virus diseases are of great importance in the cultivation of leguminous crops, extensive, systematic studies are carried out on them at the Institute of Phytopathological Research, Wageningen. The aims are to identify and record the viruses involved. A list of legume viruses already isolated in the Netherlands is given.

Stress is laid on the need for international cooperation in the study of virus diseases of crops, and of legumes in particular. The article is an introductory one of a series of publications dealing with the various viruses found in the Netherlands and the diseases caused by them.


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DRUK: H. VEENMAN EN ZONEN N.V. – WAGENINGEN

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T. Pl.ziekten 65 (1959): 13–23

# THE RED CLOVER VEIN-MOSAIC VIRUS IN THE NETHERLANDS[1]

*Met een samenvatting:*
*Het nerfmozaïekvirus van rode klaver in Nederland*

BY

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## INTRODUCTION

The red clover vein-mosaic virus was first discovered and described by OSBORN (1937) in the United States. WEISS (1939) designated the virus as *Trifolium virus 2* OSBORN. HAGEDORN & HANSON (1951) established the relationship between this virus and the incitant of the "Wisconsin pea stunt" disease described by HAGEDORN & WALKER (1949).

GRAVES & HAGEDORN (1956) reported that the red clover vein-mosaic was very widespread in Wisconsin among several naturally occurring leguminous plants and also in hay fields and pastures which contained substantial amounts of red clover, *Trifolium pratense* L. Recently JAMALAINEN (1957) noted a disease in Finland similar to the red clover vein-mosaic. MATSULEVICH (1957) reported this disease in red clover in Ukraine, U.S.S.R. In a field survey of pea diseases in Western Europe made during the summer of 1957 HAGEDORN (1958) stated the occurrence of the virus in peas, *Pisum sativum* L., in The Netherlands, Switzerland, England and Sweden. QUANTZ (1958) observed the pea stunt disease in several pea growing areas in Western Germany and isolated the virus from red clover and peas. According to a survey of virus diseases by KOCHMAN & STACHYRA (1958) the red clover vein-mosaic is also present in Poland.

Symptoms produced by this virus in clovers in general are characterized by vein clearing and vein chlorosis often together with some chlorosis of very few irregularly bordered, adjacent leaf tissue ("vein mosaic"). Diseased peas also show such symptoms but more peculiarly are very much stunted in size, with the apical foliar and inflorescent growth malformed and congested into a terminal rosette. These very characteristic symptoms help immeasurably in field diagnosis of the virus. Pea plants with symptoms suspected of being those incited by the red clover vein-mosaic virus have been observed in The Netherlands for several years. In 1957, the senior author and Ir. N. HUBBELING observed such pea plants near Wageningen (HAGEDORN, 1958) and later in the growing season the authors discovered several red clover plants in the nearby vicinity showing typical symptoms of the vein-mosaic disease (fig. 1). These plants were taken to the greenhouse and eventually one (RK5) was chosen for more detailed study. The virus isolated from this plant has been compared with Wisconsin isolates. This paper reports the results of this study, which also includes a serological comparison with an American isolate. Also in 1958 the virus was isolated several times from red clover, peas and white clover, all naturally infected.

[1] Accepted for publication 1 December, 1958.
[2] University of Wisconsin National Science Foundation Senior Post-Doctoral Fellow.

## MATERIALS AND METHODS

The greenhouse studies were conducted at temperatures ranging from 18 to 23 °C under insect-proof conditions. No artificial light was used. The plants were grown in sterilized compost soil contained in either 4- or 6-inch clay pots.

Most of the experiments were carried out either two or three times in order to make results more meaningful. Appropriate controls were provided.

The physical property tests were conducted in a manner similar to that described by JOHNSON & GRANT (1932).

For the electron micrograph study, the microscope grids were covered with a thin layer of collodion. Virus-infected plant sap was applied to the grids in two different ways:

1. About 50 mg. of infected plant material was ground in a mortar together with 2.5 ml. of distilled water. The sap thus obtained in a dilution of about 1 : 50 and in a dilution of 1 : 100 was atomized onto the grids by using a pyrex nebulizer.
2. The cut-surface of a diseased leaf was dipped for 1 to 2 sec. in a small drop of distilled water on top of the grid according to the method developed by BRANDES (1957). Afterwards in most cases a suspension of about 0.01 % solids of polystyrene latex with calibrated particles of 340 mμ diam. was sprayed onto the grids to serve as a standard for the magnification of the electron microscope. We did not observe serious deviations in form and size as noticed by BRANDES & PAUL (1957). The grids prepared in this way were shadow cast with palladium, and viewed with a Philips electron microscope.

The plant sap used for producing an antiserum was prepared as follows. Sap from deep-frozen diseased peas was centrifuged for 20 min. at 10,000 r.p.m. The supernatant was then centrifuged for one hour at 39,460 r.p.m. on top of a 0.5 ml. layer of a 40 % sucrose solution. The resulting layer in the border region between the plant sap and the sucrose solution was used for injection. Inoculation experiments had shown that the bulk of the virus was present in this region. One rabbit was interveinously injected in the ear six times in total during two weeks with quantities of the virus concentrate gradually increasing from 1 to 5 ml. The blood was taken four days after the last injection. Another rabbit was injected four times in one week, each time with 2 to 5 ml. of the virus concentrate. Since the state of the ear did not permit further injections and the titer of the serum was still too low, 10 and 14 days respectively after the last injection an intramuscular injection with an emulsion of 2 ml. virus concentrate and 2 ml. of a mixture of paraffin- and lanolin oil was given in the upper hind leg according to the adjuvant technique described by FREUND (1947). This rabbit was bled seven days after the last injection. Serological tests were performed according to the method of the micro-reaction under paraffin oil developed by VAN SLOGTEREN (1955). The sap of diseased and of healthy deep-frozen peas to be tested was at first centrifuged for 30 min. at 10,000 r.p.m. in order to remove crude constituents. Before using the antiserum, this was mixed with sap of healthy peas. This mixture was stored during one night at 0 °C and then one hour at 25 °C and subsequently centrifuged for 20 min. at 15,000 r.p.m. The dilutions of both antiserum and plant sap were made with distilled water.

In the insect-transmission test pea plants, variety Mansholt's Pluk, were used

as a source of virus and as test plants. The aphid species tested were the green pea aphid, *Acyrthosiphon pisum* HARRIS, the green peach aphid, *Myzus persicae* SULZ., and the black bean aphid, *Aphis fabae* SCOP. After a starvation period of one hour, the feeding period on diseased plants varied from 5 min. to 1 hr. Then the aphids were transferred to the healthy test plants, five aphids per plant in the first preliminary trial, 10 to 15 aphids per plant in further experiments. On these plants they fed three to six days in the first trial and 24 hrs. in the further experiments. Then they were killed with an insecticide. The plants were kept under observation in the insect-proof greenhouse.

## EXPERIMENTAL RESULTS

*Host Range.* Fourteen potential host plants were tested for their reaction to isolate RK5. The plants studied and results obtained are given in table 1 together with the reaction of the Wisconsin pea stunt isolate of the red clover vein-mosaic virus as reported by HAGEDORN & WALKER (1949) and HAGEDORN & HANSON (1951). In most cases, inoculations back to healthy peas were made to prove the presence of the virus.

These experiments indicate the RK5 virus isolate from The Netherlands has a host range which is identical to the host range reported for the red clover vein-mosaic virus isolates pea stunt and 101 studied in Wisconsin. Median red clover was not tested in Wisconsin but in our test it developed typical symptoms. According to HAGEDORN & WALKER (1949) in Wisconsin the Ladino white clover was found not to be susceptible. GRAVES & HAGEDORN (1956), however, were able to isolate the virus from naturally infected Ladino clover. It is significant to note that only legumes became diseased and that bean, *Phaseolus vulgaris* L., was not found to be a host.

By means of back inoculation from species previously inoculated with another isolate of the virus *Crotalaria spectabilis* ROTH., *Lathyrus odoratus* L., and *Ornithopus sativus* BROT. were shown also to act as host plants. They have not been recorded as such before.

*Pea Varietal Reaction.* Eighteen pea varieties, 11 from the United States and seven from The Netherlands, were tested for their reaction to the RK5 isolate of the red clover vein-mosaic virus. All varieties were susceptible. They included Alaska, Deep Green Perfection, Early Perfection, Freezer 37, New Era, New Season, New Wales, Perfected Wales, Superior, Surprise, Thomas Laxton, Koroza, Kreuk Erwt, Rondo, Servo, Unica, Vares, and Zelka. The results agree very favorably with those reported for American isolates of the virus.

*Symptomatology.* Greenhouse-grown red clover plants which were inoculated with virus isolate RK5 began to show the typical vein clearing symptoms about one month after inoculation. The intensity of vein chlorosis continued until, after about two more weeks, this symptom had developed into the characteristic conspicuous vein yellowing together with a slight vein mosaic (fig. 1). This was the dominant symptom noted and it was identical to the symptom previously observed on red clover plants inoculated with American isolates of the virus. Sometimes the leaves were slightly distorted. It was observed in the greenhouse that the symptoms in red clover often varied in severity during the development of the plant. Generally the plants appeared to suffer only slightly.

Diseased sweet clover, *Melilotus* spp. (fig. 1), white Dutch clover, *Trifolium*

TABLE 1. Host range studies with the red clover vein-mosaic virus.
*Waardplantonderzoek met het nerfmozaïekvirus van rode klaver.*

| Host *Waardplant* | | Reaction to isolate[1] *Reactie op isolatie*[1] | |
|---|---|---|---|
| Scientific name *Wetenschappelijke naam* | Common name *Naam* | Dutch RK5 *uit Nederland* | Wisc. Pea Stunt[2] *uit Wisconsin*[2] |
| *Melilotus albus* MED. | White Blossom Sweet Clover *witte honingklaver ras White Blossom* | + | + |
| *Melilotus albus* MED. var. *annua* | Hubam Sweet Clover *witte honingklaver ras Hubam* | + | + |
| *Melilotus officinalis* LAM. | Sweet Clover *gele honingklaver* | + | + |
| *Phaseolus vulgaris* L. | Bean *boon* | — | — |
| *Pisum sativum* L. | Pea *erwt* | + | + |
| *Trifolium incarnatum* L. | Crimson Clover *inkarnaatklaver* | + | + |
| *Trifolium pratense* L. | Median Red Clover *rode klaver ras Median* | + | 0 |
| ——— | Wis. Mildew Resistant Red Clover *rode klaver ras Wis. Mildew Resistant* | + | + |
| *Trifolium repens* L. | White Dutch Clover *Hollandse witte klaver* | + | + |
| *Trifolium repens* L. forma *giganteum* | Ladino Clover *Ladino witte klaver* | + | — |
| *Vicia faba* L. | Broad bean *tuinboon* | + | + |
| *Cucumis sativus* L. | Cucumber *komkommer* | — | — |
| *Nicotiana glutinosa* L. | | — | 0 |
| *Nicotiana tabacum* L. | White Burley Tobacco *White Burley tabak* | — | — |

[1] + = susceptible *vatbaar* — = not susceptible *niet vatbaar* 0 = not tested *niet getoetst*

[2] according to data published by HAGEDORN & WALKER (1949) and by HAGEDORN & HANSON (1951).
*naar gegevens van* HAGEDORN & WALKER (1949) *en* HAGEDORN & HANSON (1951).

*repens* L. (fig. 1), and Ladino white clover, *Trifolium repens* L. *giganteum*, showed only the vein chlorosis/vein mosaic symptom reported as typical of the red clover vein-mosaic virus on these hosts. This symptom, and also leaflet curling and plant stunt, were observed on diseased plants of crimson clover, *Trifolium incarnatum* L. These symptoms are considered typical of this virus on this host. Infected broad bean plants, *Vicia faba* L., showed a stunting of the top together with a distinct vein chlorosis (fig. 2). Generally these plants wilted after some time.

The red clover vein-mosaic virus incites a disease called "pea stunt" in the United States. This name is very descriptive of the over-all effect of this virus on pea plants which become infected when young. Pea plants developed this plant stunt upon inoculation with virus isolate RK5 (fig. 3). Other symptoms

reported for this virus on pea include 1. vein clearing and chlorosis, 2. downward curling of the leaflets and stipules, 3. failure of internodes to elongate properly resulting in the formation of an apical rosette of leaves, stipules, and sometimes blossoms, and 4. premature death. Most, if not all, of these symptoms were observed on almost all of the pea varieties which became infected upon inoculation with isolate RK5.

*Physical Properties.* The physical properties, tolerance to dilution, thermal inactivation, and resistance to aging, of isolate RK5 were studied in the laboratory and greenhouse. The dilution endpoint turned out to be between 1 : 1,000 and 1 : 10,000, whereas virus was still active after a ten minutes' exposure to 60° but not to 65° C. The aging *in vitro* studies were at first negative, even for one day aging but in later experiments once after one day aging virus was still active.

According to HAGEDORN & WALKER (1949) and HAGEDORN & HANSON (1951) the data for Wisconsin isolates are: dilution endpoint between 1 : 10,000 and 1 : 100,000, thermal inactivation point between 55° and 60°C, and aging *in vitro* between one and two days (HAGEDORN & WALKER) and two and three days (HAGEDORN & HANSON).

These results suggest a striking similarity between RK5 and the American (Wisconsin) isolates of the red clover vein-mosaic virus. In the dilution tests the difference is a slight one, infection with the American isolates at a dilution 1 : 10,000 being obtained only once in one plant (HAGEDORN & HANSON, 1951).

*Electron Microscopy.* The electron micrographs revealed particles consisting of thin, rigid rods (fig. 4). They were observed and studied in diseased pea, broad bean, red and white clover and in sweet clover.

In the first micrographs the distribution curves demonstrated two peaks (fig. 5B); often there were some smaller intermediate peaks (fig. 5 B, D, E). This suggested a relation between particles of different lengths and a tendency of the red clover vein-mosaic virus particles to break into two, three, four or sometimes more parts. This was supported by the observation that often a number of small particles, which together had the length of the assumed virus unit, were lying close together. The shorter particles were found in all host plants studied. So far the reason for this breakage is unknown. The method of applying the particles to the grids may play an important role. E.g. the sap used for making the preparations which produced the data represented in the curves B, D, and E (fig. 5) was prepared by grinding diseased plant material in a mortar and then sprayed onto the grids. The particles of C, on the other hand, were put onto the grids by means of the dip-method; in this way they were handled very carefully. Using the latter method there was less tendency to break or none at all. Curve A represents particle lengths in the electron micrographs of diseased sap pretreated as described on p. 14 and then mixed with normal serum (e.g. fig. 6, above). Using the dip-method in broad bean sap once there was also a clear tendency of the particles to aggregate to rigid threads of two or three times the assumed basic length (fig. 5C).

A summary of a number of particle length distributions is given in fig. 5. They show that the basic particle length is between 600 and 700 m$\mu$. Since the reliability of our measurements is not high enough, we cannot calculate the standard length of the particles with great accuracy. The magnification of the electron microscope used was not always exactly the same, and polystyrene balls were not always added as a control. Moreover the image of the particles showed

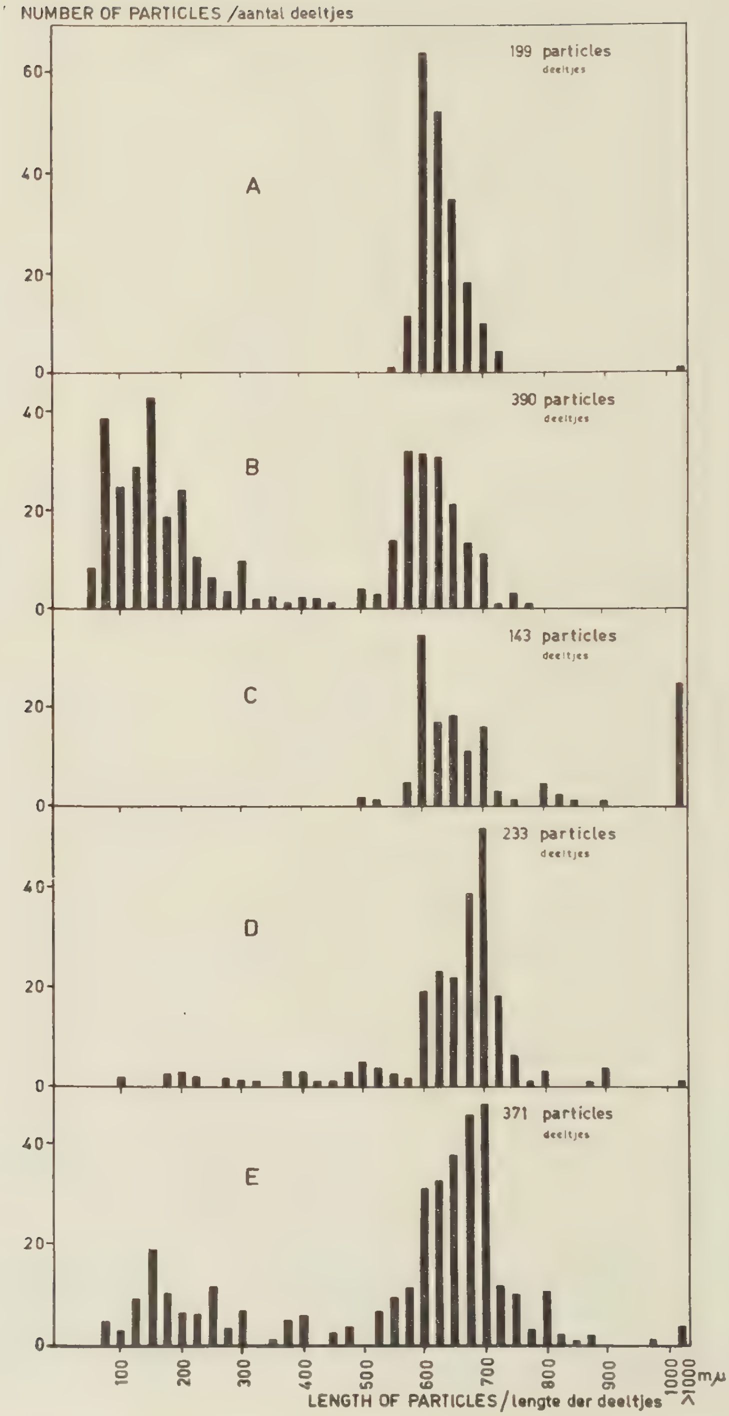
NUMBER OF PARTICLES /aantal deeltjes
199 particles
deeltjes
A
390 particles
deeltjes
B
143 particles
deeltjes
C
233 particles
deeltjes
D
371 particles
deeltjes
E
LENGTH OF PARTICLES / lengte der deeltjes
mμ

FIG. 5.

FIG. 1. Symptoms produced by the Dutch isolate RK 5 of the red clover vein-mosaic virus on the leaves of red clover (upper row), white clover (middle row) and yellow sweet clover (lower row). Right, healthy leaves.
*Symptomen veroorzaakt door de Nederlandse isolatie RK 5 van het nerfmozaïekvirus van rode klaver in de bladeren van rode klaver (boven), witte klaver (midden) en gele honingklaver (onder). Rechts, gezonde bladeren.*

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FIG. 5. Distribution curves of the lengths of specific particles present in different host plants infected with the red clover vein-mosaic virus; *A*, *B* peas, *C* broad bean, *D* red clover, *E* white clover.
*Frequentiekrommen der lengten van de specifieke deeltjes voorkomend in verschillende plantesoorten besmet met het nerfmozaïekvirus van rode klaver*; A, B *erwten*, C *tuinboon*, D *rode klaver*, E *witte klaver*.

FIG. 2.

Symptoms produced by the Dutch isolate RK 5 of the red clover vein-mosaic virus on broad bean leaves. Left, diseased leaf; right, healthy leaf.

*Symptomen veroorzaakt door de Nederlandse isolatie RK 5 van het nerfmozaïekvirus van rode klaver op de bladeren van tuinboon. Links, ziek blad; rechts, gezond blad.*

FIG. 3.

Pea, variety Unica, with pea stunt caused by the Dutch isolate RK 5 of the red clover vein-mosaic virus. Left, diseased plant; right, healthy plant.

*Erwt, ras Unica, met gedrongen top tengevolge van infectie met de Nederlandse isolatie RK 5 van het nerfmozaïekvirus van rode klaver. Links, zieke plant; rechts, gezonde plant.*

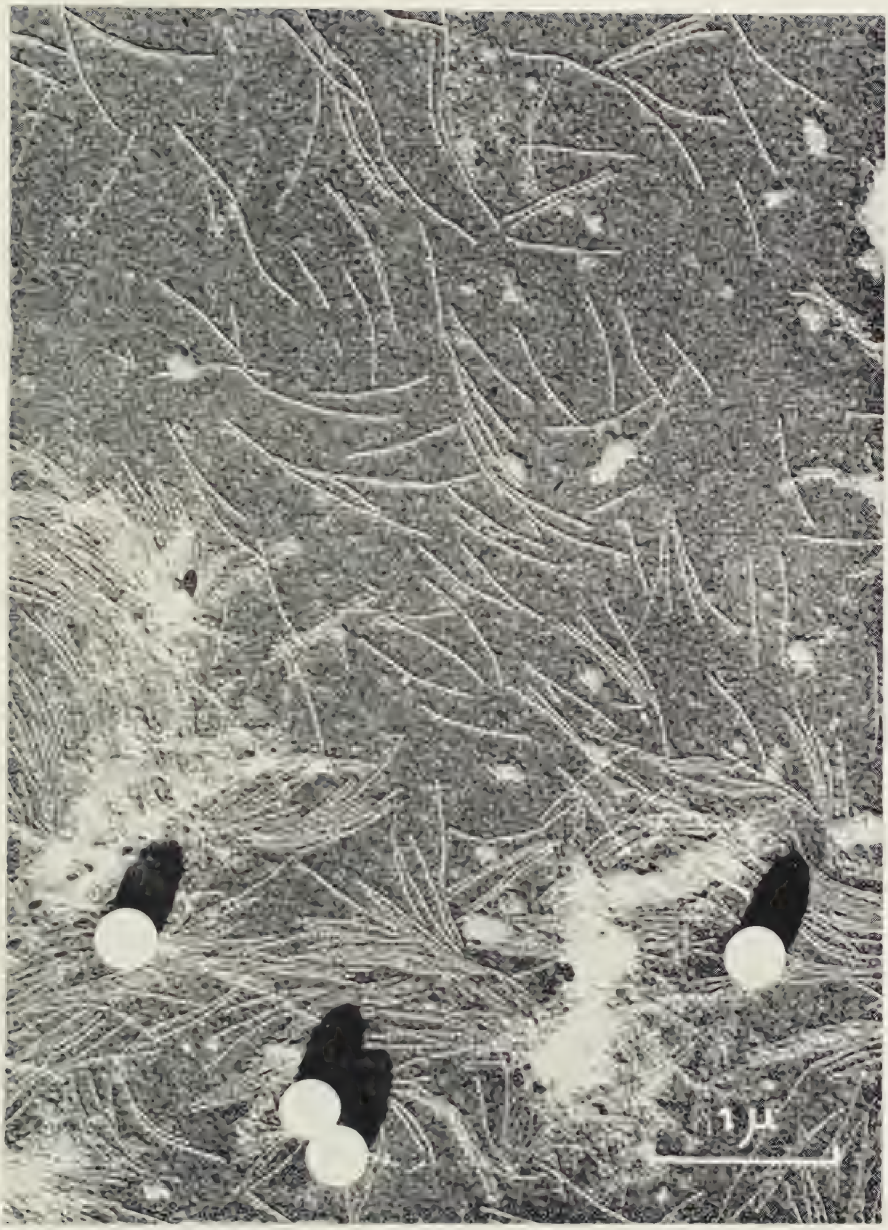


FIG. 4. Electron micrograph of the specific particles in pea infected with the red clover vein-mosaic virus; preparation made according to the dip-method. Magnification × 24,000. (Photograph made by the Physico-Technical Service for Agriculture, Wageningen.)

*Elektronenmicroscopische opname van de specifieke deeltjes voorkomend in sap van een erwteplant besmet met het nerfmozaïekvirus van rode klaver; preparaat gemaakt volgens de indoopmethode. Vergr. 24.000 ×. (Foto gemaakt door Stichting Landbouw Fysisch-Technische Dienst, Wageningen.)*

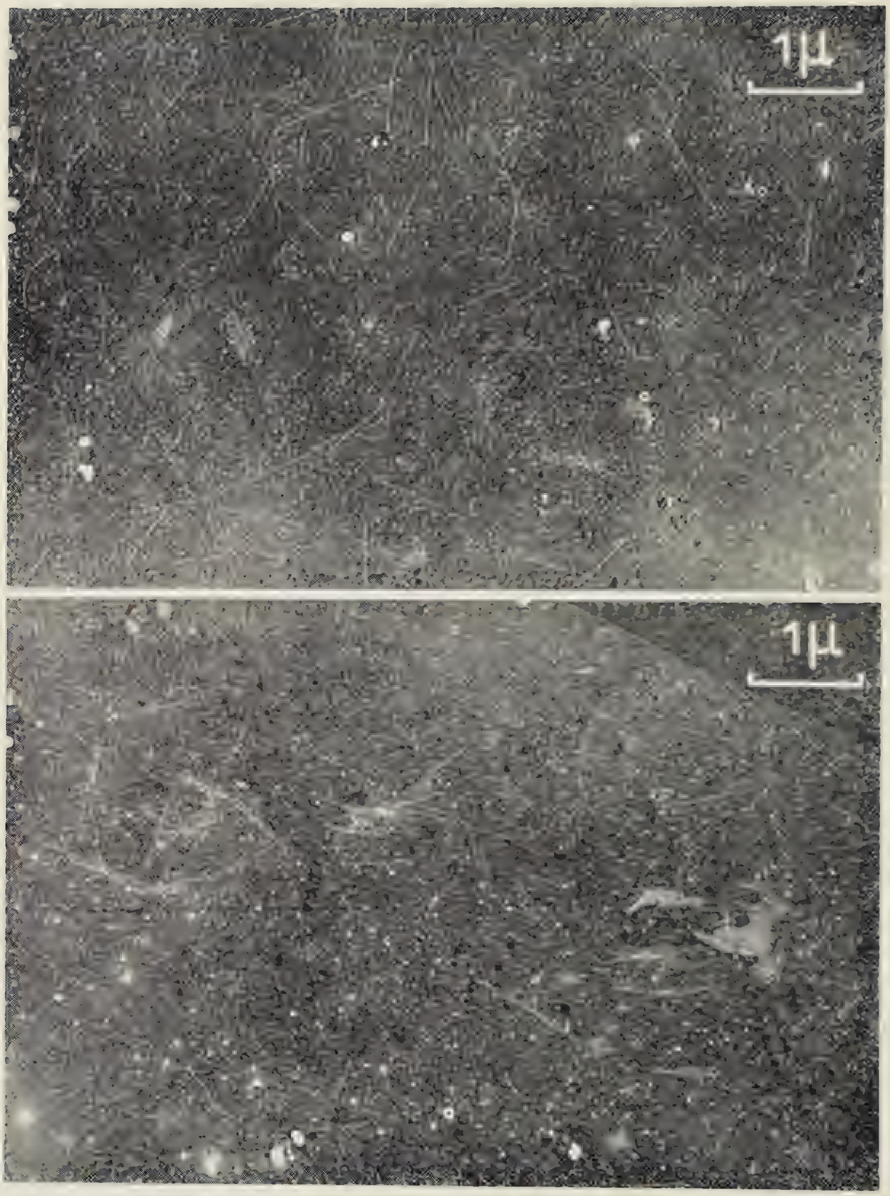


FIG. 6. Electron micrographs of the flocculating effect of the antiserum on the particles characteristic for the red clover vein-mosaic. Sap of diseased peas mixed with normal serum (above) and with antiserum (below). Magnification × 15,000. (Photographs made by the Physico-Technical Service for Agriculture, Wageningen.)

*Elektronenmicroscopische opnamen van het uitvlokkende effekt van antiserum op de deeltjes, welke karakteristiek zijn voor het nerfmozaïek van rode klaver. Sap van zieke erwten gemengd met normaal serum (boven) en met antiserum (onder). Vergr. 15.000 ×. (Foto's gemaakt door Stichting Landbouw Fysisch-Technische Dienst, Wageningen.)*

an aberation in dimensions which was more pronounced the greater the distance of the particles from the center of the field.

*Serology*. In testing the antiserum we used a mixture of antiserum and sap of healthy peas as a control. This sap and the sap of the infected plants was pretreated as described on page 14.

At first the antiserum was tested separately against the Dutch isolate and the Wisconsin isolate at Wageningen and Madison, Wisconsin, respectively. Both gave a clear positive reaction. To make results more meaningful and entirely comparable, the Wisconsin isolate of the virus sent to Wageningen and the Dutch isolate were tested simultaneously. The scheme and the results of this test are given in table 2. The serological reaction was positive and very clear.

TABLE 2. Reaction of the Dutch isolate RK 5 and a Wisconsin isolate of the red clover vein-mosaic virus with the antiserum prepared against the Dutch isolate.
*Reactie van de Nederlandse isolatie RK 5 en een isolatie van het nerfmozaïekvirus van rode klaver uit Wisconsin met het antiserum bereid tegen de Nederlandse isolatie.*

| | dilutions of sap of peas *verdunningen van sap van erwten* | antiserum *antiserum* | | | | normal serum *normaal serum* | saline *fysiologisch zout* |
|---|---|---|---|---|---|---|---|
| | | 1/4 | 1/16 | 1/64 | 1/256 | | |
| Dutch isolate RK 5 | 1/4 | +++[1] | ++ | ++ | — | — | — |
| *Nederlandse isolatie* | 1/16 | ++ | + | + | — | — | — |
| *RK 5* | 1/64 | —[2] | — | — | — | — | — |
| Wisconsin isolate | 1/4 | +++ | +++ | ++ | + | — | — |
| *isolatie uit Wisconsin* | 1/16 | ++ | ++ | + | | | — |
| | 1/64 | — | — | — | — | — | — |
| healthy pea | 1/4 | — | — | — | — | — | — |
| *gezonde erwt* | 1/16 | — | — | — | — | — | — |
| | 1/64 | — | — | — | — | — | — |

[1] The number of + signs is an indication for the quantity of precipitate.
*Het aantal + tekens is een maat voor de hoeveelheid precipitaat.*
[2] No precipitate.
*Geen precipitaat.*

The results provide definite evidence that the Dutch isolate and the Wisconsin isolate are very similar.[1]

After these results pretreated sap of diseased and of healthy peas was diluted 1 : 4 and each put into two narrow glass tubes and mixed with the same quantity of a) antiserum and of b) normal serum, both diluted 1 : 4. After 30 min. at 37 °C only in the combination diseased sap with antiserum, was a serological reaction visible. Then the material in the tubes was diluted 1 : 2 and some of it sprayed onto grids and viewed in the electron microscope. The results are pictured in fig. 6. These electron micrographs clearly demonstrate that the anti-

[1] Dr. C. WETTER, Institut für Serologie, Biologische Bundesanstalt, Brunswick, Germany, to whom the authors are greatly indebted, was so kind to test their antiserum against the German pea stunt virus, "Stauchevirus der Erbse" (QUANTZ, 1958). The positive reactions obtained, demonstrate the relationship between the Dutch and the German isolates of the red clover vein-mosaic virus. In the same way a German antiserum, tested bij the authors against the Dutch RK 5, reacted positively.

serum has a flocculating effect on the particles characteristic for the red clover vein-mosaic and for pea stunt.

*Insect Transmission.* In a preliminary trial with the green pea aphid, *Acyrthosiphon pisum*, after one hour starvation and a subsequent infection feeding of half an hour and one hour, respectively, six out of eight and four out of eight plants were infected. The results of another experiment with three aphid species and varying infection-feeding periods are summarized in table 3. In a number of cases infection of test plants was checked by means of back inoculation to peas, variety Mansholt's Pluk.

TABLE 3. Insect transmission of the red clover vein-mosaic virus.
*Overdracht van het nerfmozaïekvirus van rode klaver door insekten.*

| aphid / *bladluis* — infection-feeding period / *zuigtijd op besmette plant* | *Acyrthosiphon pisum* plants infected / plants tested (*geïnfecteerde planten / getoetste planten*) | *Acyrthosiphon pisum* back inoculation (*terug-inoculatie*) | *Myzus persicae* plants infected / plants tested (*geïnfecteerde planten / getoetste planten*) | *Myzus persicae* back inoculation (*terug-inoculatie*) | *Aphis fabae* plants infected / plants tested (*geïnfecteerde planten / getoetste planten*) | *Aphis fabae* back inoculation (*terug-inoculatie*) |
|---|---|---|---|---|---|---|
| 5 min. | 2/8 | + | 0/8 | | 0/8? | — |
| 10 min. | 2/8 | + | 1/8[1] | + | 0/8 | |
| 15 min. | 1/8[1] | + | 0/8 | | 0/8 | |
| 30 min. | 0/8 | | 0/8? | + | 0/8 | |

[1] Presence of the virus was also proved by means of the electron microscope.
*Aanwezigheid van het virus werd ook bewezen door middel van de elektronenmicroscoop.*

The results obtained indicate that at least *Acyrthosiphon pisum* and *Myzus persicae* can act as a vector and that the virus is of the non-persistent type.

The ability of the pea aphid to transmit the virus was first demonstrated by OSBORN (1937). This aphid is also the vector in Wisconsin (HAGEDORN & WALKER, 1949; HAGEDORN & HANSON, 1951). According to GRAVES & HAGEDORN (1956) transmission of the virus by the small clover aphid, *Myzocallis ononidis* KALT., also might help to explain virus movement in the field.

## DISCUSSION

The discovery of the red clover vein-mosaic virus in The Netherlands is important from several standpoints. It provides added evidence that individual plant viruses have a wide geographical distribution. The research reported here was done independently from the results of QUANTZ (1958) in Germany published recently. His publication and this one are the first known experimentally proven reports of the occurrence of this virus in Western Europe. HAGEDORN (1958) has previously reported field diagnosis of this virus in peas in The Netherlands, Switzerland, England, and Sweden. The presence of this virus in

The Netherlands means that there is the potential for the development of the pea stunt disease which is caused by the red clover vein-mosaic virus. In certain instances, this disease has been troublesome in Wisconsin.

The striking similarities between the results of experiments with the Dutch virus isolate RK 5 including studies of symptoms, host range, and serological tests, and those reported in the United States of America for the red clover vein-mosaic virus provide sufficient evidence for the conclusion that RK 5 is indeed the red clover vein-mosaic virus. The pea varietal reaction studies and the symptoms produced in these varieties also support this conclusion.

The relation of the Dutch and the American isolates of this virus to the German "Stauchevirus" (stunt virus) isolated from naturally infected peas and red clover (QUANTZ, 1958) is not entirely clear. There seem to be some differences. On a number of known host plants, such as broad bean, crimson clover, Ladino white clover and sweet clover, QUANTZ after mechanical inoculation, only observed a latent infection. Moreover in Germany in peas often top wilting and necrotic, "streak-like" symptoms appeared. Therefore QUANTZ suggested some relation with the "Wisconsin pea streak" virus. Only in a few cases in our greenhouse experiments with peas some internal necrosis of veinlets and stems was observed. The results of the German thermal inactivation test (complete inactivation between 60 and 65 °C) were similar to ours with the Dutch RK 5. The dilution endpoint of the German isolates however (between 1 : 100,000 and 1 : 1,000,000) is higher than of the Dutch isolate (1 : 1000–1 : 10,000) and the Wisconsin isolates (1 : 10,000–1 : 100,000). These facts suggest a slight difference between the virus, described by QUANTZ on one hand and those described in the U.S.A. and in this publication on the other hand.[1]

The electron microscope studies provided interesting and helpful information with regard to the morphology of the particles associated with red clover vein-mosaic virus infection. Noteworthy is the fact that the rigid rods easily break into smaller particles and sometimes aggregate into longer ones. Presumably the breakage can be prevented by making the preparations according to the dip-method.

This is the first known report on serological activity of the virus. Electron micrographs give clear evidence of the specificity of this reaction.

## SUMMARY

The presence in The Netherlands of the virus of red clover vein-mosaic and pea stunt is proven for the first time. Experimental evidence to substantiate the identification of the virus is presented including data on host range, pea varietal reaction, symptomatology and physical properties. New artificial host plants are *Crotalaria spectabilis* ROTH., *Lathyrus odoratus* L., and *Ornithopus sativus* BROT.

This is the initial publication of electron microscope photographs of particles characteristic for the above mentioned diseases. They appear to have a length between 600 and 700 mμ, to be thin and rigid and to break easily into smaller rods.

An antiserum against the Dutch isolate of the virus was prepared. Both the

[1] Serologically the German isolate is related to the Dutch one, see footnote page 19.

Dutch and the Wisconsin isolate reacted similarly.[1] The flocculating effect of this antiserum on the virus particles was demonstrated and pictured by means of the electron microscope.

It was proven that the green pea aphid, *Acyrthosiphon pisum* HARRIS, and the green peach aphid, *Myzus persicae* SULZ., are able to act as vectors of the virus, and that the virus is of the non-persistent type.

SAMENVATTING

Het voorkomen van het nerfmozaïekvirus van rode klaver in Nederland werd bewezen. Dit virus is ontdekt in de Verenigde Staten van Amerika (OSBORN, 1937) en komt in Wisconsin zeer verspreid voor in verschillende gekweekte en wilde vlinderbloemigen (GRAVES & HAGEDORN, 1956). Het veroorzaakt daar tevens een ernstige ziekte in erwten, bekend als „pea stunt" (HAGEDORN & WALKER, 1949; HAGEDORN & HANSON, 1951). Ook in Oost Europa (JAMALAINEN, 1957; MATSULEVICH, 1957), in Duitsland (QUANTZ, 1958) en in Polen (KOCHMAN & STACHYRA, 1958) is het virus onlangs gerapporteerd. Het virus werd door ons in Nederland geïsoleerd uit natuurlijk geïnfecteerde erwten, rode klaver en witte klaver.

Op grond van onderzoek naar de waardplantenreeks (tabel 1), de ziekteverschijnselen (fig. 1 tot 3), de fysische eigenschappen en de serologie (tabel 2) wordt het in Nederland voorkomende virus identiek geacht met het Amerikaanse.

De symptomen in klavers variëren in het algemeen van een lichtere kleur van de nerven tot een zeer opvallende vergeling van nerven of nerfgedeelten en soms iets van het aangrenzende weefsel, waarom van nerfmozaïek wordt gesproken (fig. 1). Het ziektebeeld is zeer karakteristiek, maar vaak verdwijnen de symptomen tijdelijk geheel of ten dele. Tuinbonen reageren soms met een lichtgroene verkleuring van de nerven, terwijl de besmette planten enigszins gedrongen blijven (fig. 2). Vaak vertonen zij in het geheel geen symptomen. Aangetaste erwten vertonen overeenkomstige verschijnselen, maar hebben bovendien zeer opvallend gedrongen toppen („pea stunt"), waarin de bladeren en steunbladeren naar beneden gekruld en enigszins gekroesd zijn (fig. 3). De planten sterven vaak voortijdig af. Met het oog op de karakteristieke gedrongenheid van de toppen van aangetaste erwteplanten, wordt als Nederlandse naam voor deze ziekte bij de erwt voorgesteld: „gedrongen-topziekte van erwt". Alle 18 getoetste erwterassen bleken vatbaar te zijn.

Als nieuwe, tot dusver nog niet in de literatuur vermelde kunstmatige waardplanten werden gevonden: *Crotalaria spectabilis* ROTH., *Lathyrus odoratus* L. en *Ornithopus sativus* BROT.

Met behulp van de elektronenmicroscoop zijn karakteristieke, betrekkelijk dunne en stugge, staafvormige virusdeeltjes waargenomen (fig. 4) met een lengte tussen 600 en 700 mμ (fig. 5).

Voor het eerst werd een specifiek antiserum bereid tegen de Nederlandse isolatie van het virus. Dit antiserum reageerde op gelijke wijze met de Nederlandse isolatie en met die uit Amerika.[2] Het uitvlokkende effect hiervan op de

[1] See footnote page 19.

[2] Het desbetreffende antiserum gaf ook positieve reacties met het Duitse „Stauchevirus der Erbse" (QUANTZ, 1958). De schrijvers zijn Dr. C. WETTER, Institut für Serologie, Biologische Bundesanstalt, Brunswijk, Duitsland, zeer erkentelijk voor de uitvoering van deze serologische proef. Een door laatstgenoemde bereid antiserum bleek, bij toetsing door de schrijvers, met de Nederlandse isolatie van het virus, eveneens zeer duidelijk positief te reageren.

voor de ziekte karakteristieke deeltjes, werd met behulp van de elektronenmicroscoop afgebeeld (fig. 6).

In kasproeven werd aangetoond dat de erwtebladluis (*Acyrthosiphon pisum* HARRIS) en de perzikbladluis (*Myzus persicae* SULZ.) het virus kunnen overbrengen (tabel 3). Het virus is non-persistent.

Het nerfmozaïekvirus van rode klaver komt waarschijnlijk in Nederland meer voor dan tot dusver werd vermoed. Gegevens over de mate van voorkomen van de ziekte en de veroorzaakte schade ontbreken nog. Gezien de in Amerika opgedane ervaring wordt ook in ons land een toenemende mate van aantasting van erwten door dit virus mogelijk geacht.


## ACKNOWLEDGEMENTS

The authors are greatly indebted to Mr. D. Z. MAAT for his permanent assistance and especially for preparing the antiserum. They also wish to express their appreciation to Miss Dra. C. VAN DER SCHEER and Mr. S. HENSTRA, Physico-Technical Service for Agriculture, Wageningen, for doing the electron microscopy.


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DRUK: H. VEENMAN EN ZONEN N.V. – WAGENINGEN